SOME ASPECTS OF STRUCTURE OF MURINE H-2\textsuperscript{d} ANTIGENS*

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The apparent molecular weight of proteins precipitated by normal rabbit serum from lysates of lymph node cells ranged from 10 000 to 150 000. Monospecific anti-H-2\textsuperscript{d} serum precipitated molecules composed of heavy and light chains with $M_r$ of 47 000 and 12 000, respectively. Tunicamycin treatment caused a decrease of the molecular weight of heavy chain by 4 000 and a change of pl from 5.2 - 5.9 to 5.4 - 5.8. Non-glycosylated heavy chain exhibited $M_r$ of 43 000. The oligosaccharide side chains were resistant to digestion by endo-$\beta$-N-acetylglicosaminidase H. The $M_r$ and pl of $\beta_2$-microglobulin were not altered by treatment with tunicamycin and endo-$\beta$-N-acetylglicosaminidase H. Tunicamycin caused a 50% decrease of overall synthesis of cellular proteins.

The murine major histocompatibility antigens H-2K and H-2D are a genetically polymorphic class of cell-surface glycoproteins. They are involved in a variety of immunological phenomena including graft rejection (for review see Klein et al., 1981; Witt, 1983). The H-2 antigen molecule is composed of two polypeptide chains, the heavy chain, an integral membrane glycoprotein bearing antigenic determinants (Schwartz et al., 1973), and non-covalently associated light chain, i.e. $\beta_2$-microglobulin (Rask et al., 1974) which is homologous with the constant region domain of immunoglobulin (Peterson et al., 1972). The H-2 antigen can be released from the cell surface by treatment with non-ionic detergents (Cunningham et al., 1973) or after proteolytic treatment with papain (Shimada & Nathenson, 1969). Although main features of H-2 antigen structure, including amino acid sequence (Coligan et al., 1981) have been elucidated, a number of properties are still unknown, e.g. the effect of tunicamycin and endo-$\beta$-N-acetyl-

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glucosaminidase H on structure of H-2, or influence of glycosylation on
the charge of H-2 molecules.

This paper presents some additional data on the structure of H-2d
antigens.

MATERIALS AND METHODS

Material. Experiments were carried out on lymph node cells of DBA/2
mice and on lymphoma cell line SL2 grown in ascites form in DBA/2 mice
(Bomholtgord, Denmark) and harvested 8 - 10 days after injection with
2 x 10^6 cells in PBS.

Chemicals. All cell culture media were purchased from DIFCO (Detroit,
U.S.A.), [35S]methionine and protein markers from New England Nuclear
(Boston, U.S.A.). Acrylamide and bis-acrylamide were purchased from B.D.H.
(Poole, England), Pharmalyte from Pharmacia (Uppsala, Sweden) and Nonidet
P-40 from Particle Data Laboratories (Elmhurst, U.S.A.). Tunicamycin was
provided by Eli Lilly (Indianapolis, U.S.A.) and endo-β-N-acetylglucosamini-
dase H by Seikagaku Kogyo Co. (Tokyo, Japan). ConcanaValin A and
Protein A-Sepharose were from Pharmacia (Uppsala, Sweden). All other
chemicals were of highest purity purchased from POCh (Gliwice, Poland).

The alloantigenic (rabbit) anti-H-2d serum, obtained by immunization
with papain-solubilized H-2d antigen (Kvist et al., 1978), was kindly
provided by Dr. P. A. Peterson (University of Uppsala).

Stimulation with ConA and radiolabelling of cells. Cells were stimulated
in Eagle’s medium enriched with 5% FCS, 6 mm-glutamine, 1% PEST
(100 U/ml penicillin, 50 μg/ml streptomycin), 5 x 10^-5 M-2-mercaptoethanol,
275 μg/ml ConA for 72 h at 37°C in the atmosphere containing 5% CO2.
Stimulated cells were suspended in methionine-free DMEM containing
5% DMEM, 6 mm-glutamine, 5% FCS (dialysed), 150 μCi/ml [35S]methio-
nine, and labelled for 4 h at 37°C in 5% CO2 at a concentration of
4 x 10^6 cells/ml. Cells (4 x 10^6) were lysed for 10 min at 0°C in 1 ml
of buffered Nonidet P-40 (0.01 M-Tris/HCl, pH 7.5, 0.15 M-NaCl, 1% NP-40,
2 mm-EDTA). Cell debris were removed by centrifugation.

Immunoprecipitation of cellular antigens. Cellular lysate (1 ml) was cleared
(preprecipitation) overnight at 4°C with 25 μl of normal rabbit serum.
The immune complexes formed were immunoabsorbed on 100 μl of 20% 
suspension of formaline-fixed, heat-inactivated Staphylococcus aureus (Cowan I
strain) in buffered NP-40 (Cullen & Schwartz, 1976). The resulting pellet
was then incubated with 5 μl of anti-H-2d serum for 4 h at 4°C and

1 Abbreviations used: NP-40, Nonidet P-40, ConA, concanaValin A; DMEM, Dulbecco
minimal essential medium; FCS, foetal calf serum; SDS-PAGE, electrophoresis in polyacryl-
amide gel with sodium dodecyl sulphate; DTT, dithiothreitol; endoglucosaminidase H, endo-
β-N-acetylglucosaminidase H; TL, thymus leukemia; HLA, human major histocompatibility
antigens; PBS, phosphate buffered saline.
immune complexes were removed as described above, washed three times with buffered NP-40, once with buffered NP-40 containing 0.5 M-NaCl and once with water. Immunoprecipitated proteins were solubilized in 100 μl of SDS-PAGE sample buffer by heating at 90°C for 3 min, alkylated with 0.05 M-iodoacetamide and subjected to electrophoresis.

**Polyacrylamide-gel electrophoresis.** Proteins were analysed by electrophoresis in 10%, polyacrylamide gel with SDS according to the modified method described by Blobel & Dobberstein (1975). After the run gels were cut into 1.5 mm slices and radioactivity was determined in a scintillation counter.

Isoelectrofocusing was performed according to the procedure described by O'Farrell (1975). After resolution of proteins in linear pH gradient 4.5 - 7.5, the electrophoresis in gradient 10 - 15%, polyacrylamide slab gels with SDS was applied (Blobel & Dobberstein, 1975). Slabs were dried, proteins detected by fluorography (Bonner & Laskey, 1974) and their molecular weight estimated by comparison with radioactive markers: phosphorylase b (M, 92 500), bovine serum albumin (69 000), ovalbumin (46 000), carbonic anhydrase (30 000), lactoglobulin A (18 400) and cytochrome c (12 300).

**Tunicamycin treatment.** Tunicamycin was dissolved in 0.01 M-NaOH and stored in small portions at a concentration of 1 mg/ml at -70°C (Takatsuki et al., 1975). The cells were preincubated with 2 μg/ml of tunicamycin for 2 h in DMEM and then labelled with [35S]methionine. During the labelling tunicamycin was present in the medium at the same concentration.

**Endo-β-N-acetylglucosaminidase H digestion.** The enzyme was dissolved in 0.02 M-Tris/HCl, pH 8.0, containing 0.15 M-NaCl and stored at a concentration of 1 U/ml at -70°C (K. Sege, L. Rask and P. A. Peterson, in preparation). Immunoprecipitates obtained by immunoprecipitation were released from immunoadsorbent by incubation for 3 min at 95°C in 50 μl of 0.02 M-Tris/HCl, pH 8.0, containing 0.15 M-NaCl, 1% SDS and 10 mM-DTT. Proteins were diluted with 9 vol. of 0.15 M-sodium citrate, pH 5.0, digested with the enzyme (final concentration 0.1 U/ml) for 16 h at 37°C, and protein was precipitated with 15%, trichloroacetic acid for 2 h at 0°C. The precipitates were washed with ether/ethanol (1:1, v/v) and subjected to SDS-PAGE.

**RESULTS**

The total pool of cellular proteins precipitated by normal rabbit serum (preprecipitation) showed a wide spectrum of molecules with apparent molecular weight ranging from 10 000 to 150 000 (Fig. 1). Repeated precipitation with normal rabbit serum (control) gave no precipitate. The precipitation with rabbit anti-H-2d serum gave two peaks of proteins with apparent molecular weight of 47 000 and 12 000, corresponding to heavy chain and β2-microglobulin (the light chain), respectively. The proportion of radioactivity of the two peaks was 1:1.
Fig. 1. Electrophoresis in 10% polyacrylamide gel of proteins immunoprecipitated from lymph node cells. After electrophoresis gels were cut into 1.5 mm slices and radioactivity was estimated. Proteins were precipitated by: \( \Delta \), normal rabbit serum (preprecipitation); \( \bullet \), anti-H-2\textsuperscript{d} serum; \( \bigcirc \), normal rabbit serum (repeated precipitation, control). Position of protein markers is shown by arrows.

For further analysis of H-2\textsuperscript{d} antigen structure tunicamycin, an inhibitor of \( N \)-glycosylation, was used (Tkacz & Lampen, 1975). After incubation with tunicamycin the \( M_r \) of the heavy chain was decreased. The molecules synthesized in the presence of tunicamycin exhibited an apparent molecular weight of 43 000 (Fig. 2A), \textit{i.e.} 4 000 less than native ones. The antibiotic did not affect \( M_r \) of \( \beta_2 \)-microglobulin.

For the characterization of carbohydrate side chain of H-2 antigen endo-\( \beta \)-\( N \)-acetylglucosaminidase H, an enzyme acting on high mannose oligosaccharides, was used. The digestion of precipitated proteins labelled for 4 h with \( [35S] \)methionine had no influence on the molecular weight either of the heavy chain or \( \beta_2 \)-microglobulin (Fig. 2B).

Proteins precipitated from cells cultured with and without tunicamycin were subjected to isoelectrofocusing followed by SDS-PAGE. Beside the difference in size, the heavy chains from the two cultures differed also in their isoelectric points. The pl of heavy chain from cells cultured without tunicamycin ranged from 5.2 to 5.9 (Fig. 3A), whereas pl of heavy chain precipitated from cells incubated with tunicamycin was somewhat narrower and ranged from 5.4 to 5.8 (Fig. 3B). Under the same experimental conditions \( \beta_2 \)-microglobulin was homogeneous with respect to molecular weight (12 000) and pl (6.8).

In addition to inhibition of glycosylation tunicamycin acted also as an inhibitor of overall synthesis of cellular proteins (Table 1). The difference in radioactivity precipitated both by normal rabbit serum and anti-H-2\textsuperscript{d} serum from lysates of cells cultured with and without tunicamycin suggested a nearly 50% decrease in overall protein synthesis.
Fig. 2. Effect of tunicamycin and endo-β-N-acetylglycosaminidase H on structure of H-2d antigen. A. Antigens were precipitated from lymph node cells preincubated with tunicamycin (2 μg/ml) and subsequently subjected to 10", SDS-PAGE. B. Antigens were precipitated from lymph node cells, digested by endo-β-N-acetylglycosaminidase H and subsequently subjected to 10", SDS-PAGE. ●, Anti-H-2d serum; ○, normal rabbit serum (control).

Table 1

Immunoprecipitation of proteins from lymph node cells incubated with or without tunicamycin

Lymph node cells (10⁷) were incubated for 2 h at 37°C in 20 ml DMEM in the presence of 2 μg/ml of tunicamycin or in DMEM without tunicamycin. Both cultures were labelled with [³⁵S]methionine and proteins were immunoprecipitated as described in Materials and Methods.

<table>
<thead>
<tr>
<th>Tunicamycin</th>
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<td></td>
<td>normal rabbit (cpm ± S.D.)</td>
<td>anti-H-2d (cpm ± S.D.)</td>
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<tr>
<td>+</td>
<td>42 650 ± 6 650</td>
<td>4320 ± 950</td>
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<td>97 860 ± 18 300</td>
<td>7730 ± 2100</td>
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Fig. 3. Influence of tunicamycin on $M_t$ and charge of H-2d antigen molecules. Antigens were precipitated from labelled SL2 cells cultured with (A) or without (B) tunicamycin and subjected to isoelectric focusing (IEF) in linear pH gradient 4.5 - 7.5 (indicated above) followed by electrophoresis in 10 - 15% polyacrylamide gel with SDS (SDS-PAGE). $M_t$ shown on the left side. Arrow downward, heavy chain; arrow upward, $\beta_2$-microglobulin. Samples of approximately 2 000 cpm were subjected to electrophoresis.

DISCUSSION

The long time of labelling of cellular proteins with \(^{35}\text{S}\)methionine (4 h) guaranteed that not only precursors but first of all mature H-2\(d\) antigens were labelled (Vitetta & Uhr, 1975). The immunoprecipitation with anti-H-2\(d\) serum showed that apparent molecular weight of heavy chain of mature H-2\(d\) antigens was 47 000. The consistently observed asymmetry of the heavy chain radioactivity peak suggested that this peak could in fact be double and contain H-2K\(^\text{a}\) (\(M\), 48 000) and slightly smaller H-2D\(^\text{d}\) (\(M\), 47 000) gene products (Kivist et al., 1977; Dobberstein et al., 1979).

For estimation of approximate size of oligosaccharide side chains tunicamycin, an inhibitor of N-glycosylation blocking the N-acetylgalactosamine transfer to dolichol phosphate (Tkacz & Lampen, 1975), was used. Apparent molecular weight of non-glycosylated heavy chains was 44 000 for H-2K\(^\text{d}\) and 43 000 for H-2D\(^\text{d}\) gene products. The reduction of size of heavy chain by about 4 000 for both H-2K\(^\text{d}\) and H-2D\(^\text{d}\) may suggest that the approximate total molecular weight of oligosaccharide chains connected with heavy chain was more or less equal to the estimated difference between molecular weight of glycosylated and non-glycosylated heavy chains; however, this conclusion needs further confirmation by use of other methods. The presented value is a little lower than estimated by other authors for H-2K\(^\text{a}\) (Rothbard et al., 1980) and H-2K\(^\text{b}\) (Nathenson & Muramatsu, 1971). The presence of oligosaccharide chains did not affect antigenic properties of H-2\(d\) because both glycosylated and non-glycosylated heavy chains were precipitated by monospecific antiserum. It is suggested that folding of the H-2 antigen molecule is not dependent on the presence of oligosaccharide side chains (Rothbard et al., 1980). Resistance to digestion with endoglycosaminidase H suggested that after 4 h of incubation all antigens precipitated by specific antiserum contained oligosaccharide side chains of complex type. It has been shown previously that the enzyme acts only on high-mannose oligosaccharide chains. These data support previous reports on the structure of oligosaccharide chains of H-2 antigens (Nathenson & Cullen, 1974). Thymus leukemia (TL) antigens after short-time labelling (1 h) were not resistant to digestion with endoglycosaminidase H (Slomski & Cohen, 1980), and after 2 h they became resistant (Rothenberg & Boyse, 1979). Thus, newly synthesized TL antigen molecules could contain immature high-mannose oligosaccharide chains, and resistance could be caused by maturation of an oligosaccharide moiety to complex type one. HLA antigens 90 min after synthesis were resistant to digestion with endoglycosaminidase H (K. Sege, L. Rask & P. A. Peterson, in preparation).

Position of \(\beta_2\)-microglobulin radioactivity peak was not altered either by tunicamycin or by endoglycosaminidase H. This indicates that \(\beta_2\)-microglobulin was not glycosylated.

On two-dimensional gel electrophoresis (with isoelectrofocusing in the first dimension) differences in isoelectric point of glycosylated and non-
-glycosylated H-2nd molecules were visualized. This supports the observation that charge heterogeneity of antigen molecules is caused by various content of polar carbohydrate residues (probably sialic acid) in oligosaccharide chain (Jones, 1977; Kvist et al., 1977). The data presented here correspond to pI values 5.85-6.5 estimated for H-2a antigen (Jay et al., 1979).

The isoelectric point of β2-microglobulin chain was 6.8, that is nearly identical with pI of H-2a β2-microglobulin estimated by Jay et al. (1979). β2-Microglobulin chain was homogeneous with respect both to Mr and charge.

As a side effect of tunicamycin treatment, the inhibition of overall protein synthesis by this antibiotic was observed. This phenomenon might be caused by a specific inhibitor of protein synthesis present in the tunicamycin preparation which could be removed by high-pressure liquid chromatography (Hickman et al., 1977). A regulatory linkage between protein glycosylation and their biosynthesis cannot be excluded (Hasilik & Tanner, 1978).

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REFERENCES


NIEKTÓRE ASPEKTY STRUKTURY MYSICH ANTYGENÓW H-2d

Streszczenie

Masa cząsteczkowa białek precytopitowanych z lizatów komórek mysich węzłów chlonnych przez normalną surowicę króliczą wyniosła 10 000 - 150 000. Monospecyficzna surowica anty-H-2d precytopitowała cząsteczki składające się z łańcuchów ciężkiego i lekkiego o masach cząsteczkowych, odpowiednio, 47 000 i 12 000. Tunikamycyna powodowała obniżenie masy cząsteczkowej łańcucha ciężkiego o 4 000 i zmianę pl z 5.2 - 5.9 do 5.4 - 5.8. Nieglikozylowane łańcuchy ciężkie miały masę cząsteczkową 43 000. Boczne łańcuchy cukrowe były odpornie na trawienie endo-β-N-acetylgalaktozaminidaza H. M, i pl β2-mikroglobulinu nie uległo zmianie pod wpływem tunikamycyny i endo-β-N-acetylgalaktozaminidazy H. Tunikamycyna powodowała obniżenie całkowitej syntezy białek komórkowych o 50%.

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